CORRELATION BETWEEN STRUCTURE AND THE BIOGENIC MECHANISMS OF CELLULOSE: NEW INSIGHTS BASED ON RECENT ELECTRON MICROSCOPIC FINDINGS

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SYNOPSIS

New techniques in electron microscopy have brought about direct evidence of the unidirectionally oriented, extended-chain structure of native celluloses, as well as detailed knowledge about widely varying microfibrillar morphology. These findings are reviewed and are correlated with the structural features of the putative cellulose synthesizing apparatus in the plasma membrane. Prospects for further understanding of the mechanism of "biocrystallization" are discussed.

INTRODUCTION

While we often refer to the abundance and importance of cellulose as a biological macromolecule, a consequent question is not so often raised; "What made cellulose so?" or "Why was cellulose chosen as the major component of plant cell walls?"

The answer seems to lie in its structure itself. In preferred models of cellulose I [1, 2], the glucoside ring is rotated ca. by 180° relative to the preceding glucoside group (2_1 helix) and two intramolecular hydrogen bonds (03-H:05' and 02'-H:06) are formed between successive glucoside rings (' denotes the next glucoside ring toward the nonreducing end). This configuration is characterized by the essentially straight, sheet-like structure of the single glucan molecule. Such sheet-like molecules would tend to align parallel with each other, and if they could be arranged in an ordered way, they would form a crystal.

There are several polysaccharides known to crystallize in the native state or artificially. Many of them, however, crystallize in the form of helices with more than two sugar residues in a pitch. Well-known examples of such helices are α-1,4, glucan (amylose) and β-1,3 glucan (callose). Unlike these glucans, cellulose crystallizes with a straight-chain conformation, which results in fibrillar structures with high density and high tensile strength.*

These properties are undoubtedly advantageous for a structural material involved in the construction of cell walls. It is likely that the adoption of cellulose as the cell wall material was a key event for the successful evolution and flourishing of terrestrial green plants. Therefore, understanding of the mechanisms of biogenesis, including the polymerization and crystallization steps is important from the biological as well as the chemical point of view.

CHAIN POLARITY

Cellulose was one of the first organic materials to which x-ray diffraction analysis was applied [3, 4]. After the unit cell axes were determined, molecules were packed into the cell to build a model of native cellulose crystal structure. The first model of native crystalline cellulose was proposed by Meyer and Mark [5]. In this model all molecules are aligned with the same sense in terms of the molecular

*A recent conformational study of possible helical structures of glucose polymers has shown that only β-1,4 and α-1,3 glucans can form 2-fold helices which are stereochemically acceptable [7].

polarity; i.e., a "parallel" structure. Subsequently, the well-known Meyer-Misch model placed the center chain in the opposite sense to the corner chain, i.e., with an "antiparallel" structure [6].

The Meyer-Misch model, however, brought about some difficulty in understanding the mechanism by which organisms produce microfibrillar cellulose. Logically, there are two possible mechanisms which can give rise to an antiparallel structure; (i) chain folding to form some kind of lamellar structure; and (ii) extended chain structure produced by two kinds of enzymes, one synthesizing the glucan chain with reducing ends ahead, and the other synthesizing in the opposite way. Yet, neither of them seemed likely to occur from physico-chemical and biochemical points of view.

Attempts to answer these questions were carried out by two groups in the 1970s, through elaborate molecular modeling analyses with the aid of computers [1, 2]. Both of these studies claimed parallel chain structure for a highly crystalline cellulose from the giant siphonocladalean green alga , *Valonia ventricosa*. The two groups then studied the structure of regenerated cellulose and both concluded antiparallel structure to be highly likely [2, 8].

Though the models provided by these x-ray studies seemed to conform with presumable mechanisms of formation for cellulose I and cellulose II, there was still room for questions because of uncertainty of the diffraction data originating from the limited sizes of crystals of the specimens. In addition, a subtle but crucial difference was found in the models of cellulose I proposed by the two groups. Thus, independent and preferably, more direct evidence for the chain arrangement in cellulose crystals has been needed. Several new findings related to this topic were published in the last few years, mainly from electron microscopy.

While significant effort is needed to determine the polarity of adjacent chains by x-ray crystallography, the polarity should be exhibited in terms of chemical structure when chain ends are exposed. Along this line, an attempt to label the reducing ends with silver particles was successfully made with *Valonia* cellulose [9]. The results showed that the cellulose has reducing ends at only one end of the fragments of the microfibril, which are microscopic single crystals; i.e., they have parallel chain structure. Figure 1 shows microcrystallites from *Valonia macrophysa* labeled with silver particles nucleated by silver-proteinate attached to reducing ends.

The same feature was visualized also by the mode of enzymatic attack onto *Valonia* cellulose [10]. The fragments of microfibrils showed a wedge-like contour after digestion by cellobiohydrolase. Because this enzyme is known to depolymerize cellulose from its nonreducing end, the observed feature demonstrated the asymmetric distribution of the nonreducing end, i.e. parallel structure.

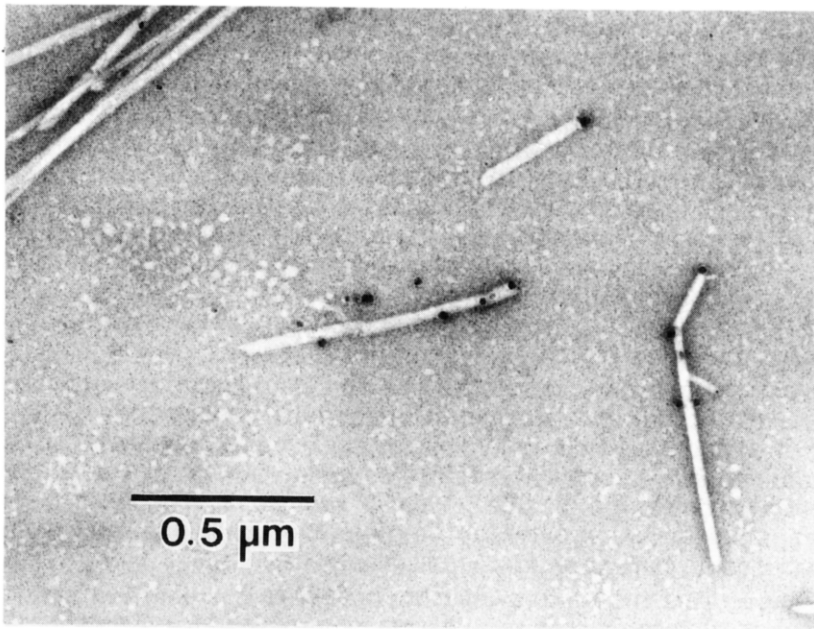

Fig. 1. Microcrystallites from *Valonia* labeled with silver at their reducing ends. (Reproduced from Ref. 11)

Subsequently, a modified technique of silver staining was applied to bacterial cellulose [11], again showing the parallel structure (Fig. 2).

The parallel structure thus confirmed for *Valonia* and bacterial cellulose conforms well with a reasonably expected mechanism of biosynthesis, such that glucan molecules are synthesized by a single kind of enzyme within the plasma membrane and then incorporated into microfibrils with extended-chain structure to form the native crystalline allomorph of cellulose I.

LATTICE IMAGES AND MORPHOLOGY OF THE MICROFIBRIL

Recently, the features of the extended chain arrangement were directly visualized by high resolution electron microscopy. The

technique of lattice imaging of cellulose was developed by combining carefully chosen beam conditions and a method of secondary enlargement from electron micrographs [12, 13]. Lattice images such as shown in Figure 3 showed high definition of lattice order and visualized the single crystalline nature of the microfibril of *Valonia*. These images also ruled out the existence of any regular longitudinal disruption of lattice order such as chain folding.

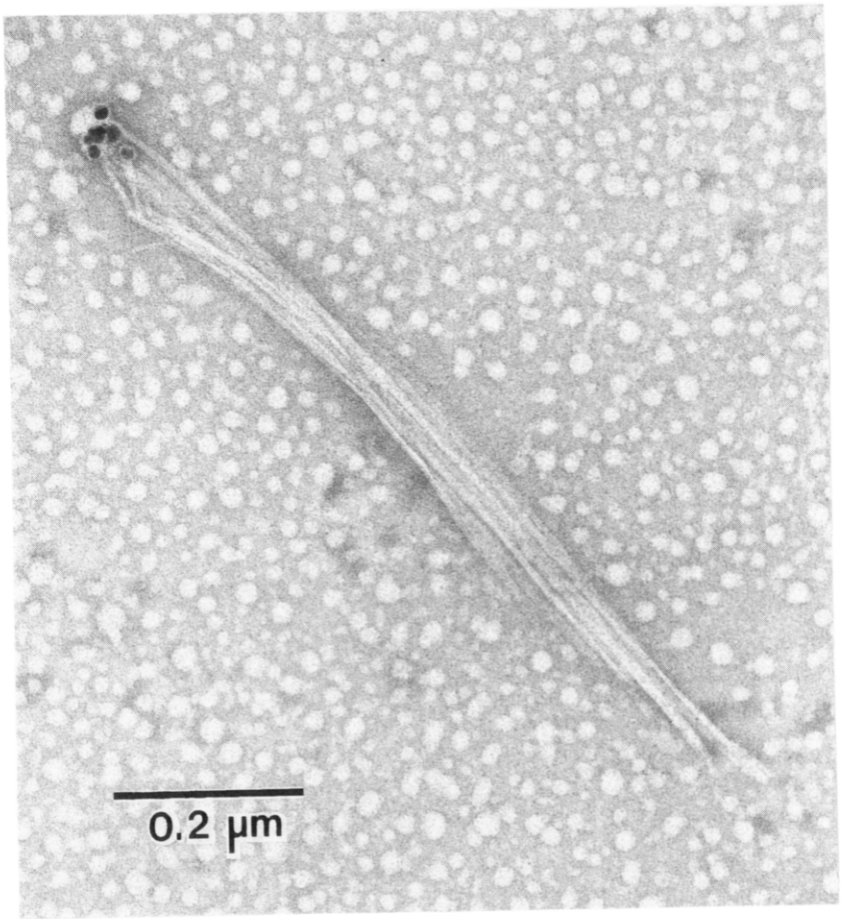

Fig. 2. Fragment of a bacterial cellulose ribbon labeled with silver at the reducing ends. (See Ref. 11 for experimental details).

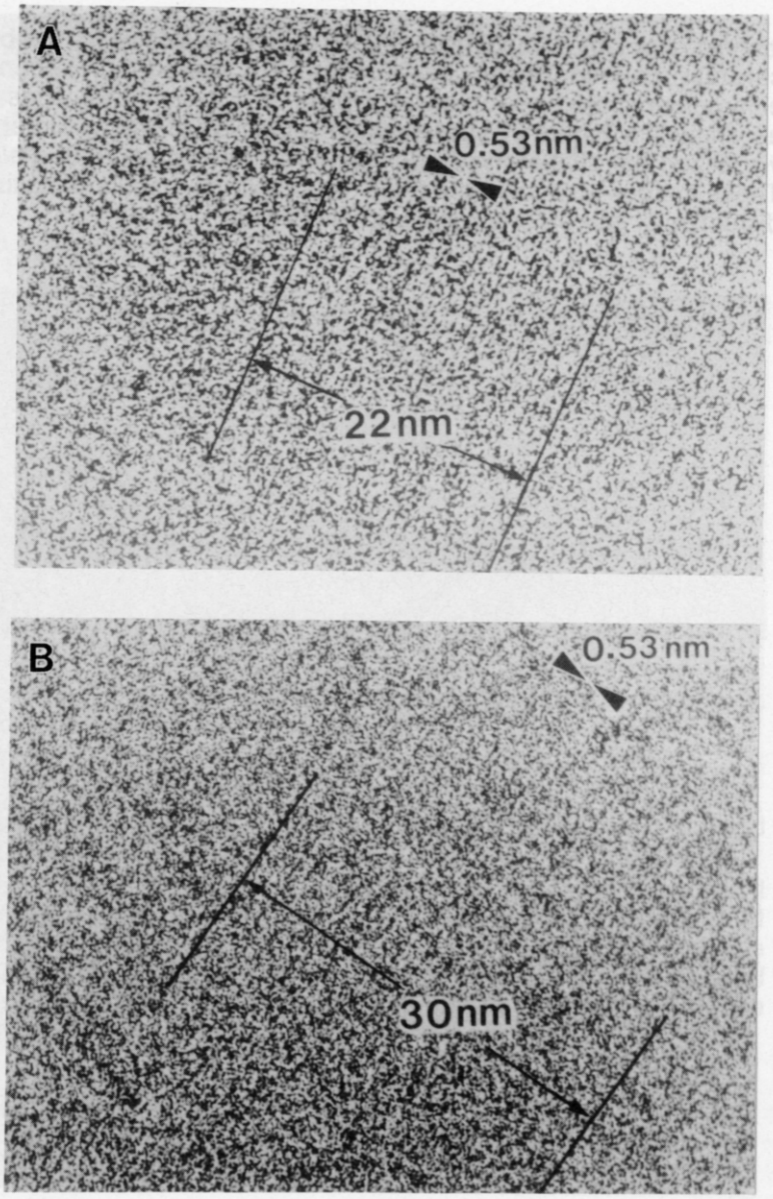

Fig. 3. Lattice images of microfibrils of (A) *Valonia macrophysa* and (B) *Boergesenia forbesii* [see 14].

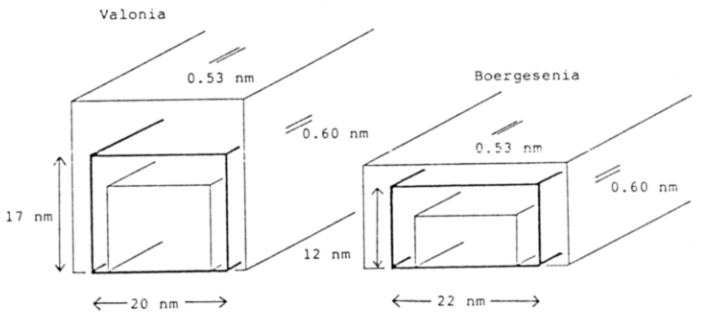

Fig. 4. Schematic drawing of cross sections of microfibrils of *Valonia* and *Boergesenia*. The horizontal base plane represents the surface of the plasma membrane. The gold frames show average sizes. The large and small frames show typical ranges of variation. Determinations were based on about 50 lattice images for each dimension. Note the same uniplanar orientation in both systems.

Lattice images were obtained also with another green alga closely related to *Valonia* [14]. Lattice images of the *Boergesenia* microfibril (Fig. 3B) demonstrated a similar feature of crystalline order, but its cross sectional shape was somewhat different from that of *Valonia*. While *Valonia*'s microfibril was found to have a nearly square cross section, *Boergesenia*'s microfibril had a significantly flattened shape. While each of the lateral dimensions show some range of variation in both *Valonia* and *Boergesenia*, average sizes were determined based on some 50 lattice images for each sample. Figure 4 is a schematic drawing of microfibrils of these algae.

Lattice images have been obtained also with microcrystalline preparations from bacterial [14] and ramie [15] celluloses. The observed lattice images were in good agreement with the crystallite sizes observed by negative staining.

CORRELATION BETWEEN MORPHOLOGIES OF THE MICROFIBRIL AND THE TERMINAL COMPLEX

Lattice images of cellulose from *Valonia, Boergesenia, Acetobacter* and ramie show that the crystalline order is continuous throughout the area of the microfibrils as observed by conventional staining

techniques. These observations appear to contradict the concept of the "elementary fibril" [16] as the smallest universal unit of native celluloses. On the contrary, this new knowledge leads us to the idea of specific control of microfibril size and crystalline morphology by the cellulose synthesizing apparatus.

The progress in the last decade in the ultrastructure of the plasma membrane has provided important clues for understanding the mechanism by which the organism controls the morphology of its cellulose microfibril. Biochemical studies of cellulose biosynthesis are providing more and more convincing evidence that the membrane-associated apparatus called the terminal complex (TC) is the synthesizing apparatus itself, or at least a structure closely conjugated to it. The shape of the TC is widely variant according to the organism. The TCs so far observed are classified as follows [17]:

(i) Linear TCs of *Acetobacter xylinum*
(ii) Linear TCs of Siphonocladalean algae
(iii) Hexagonal arrays of rosettes in Zygnematalean algae
(iv) Isolated rosettes of vascular plants

Significantly, structural features of cellulose seem to have a close correlation with the type of TC. Figure 5 shows a schematic drawing of microfibrils and corresponding TCs. It is evident that the formation of a highly crystalline microfibril requires a regular arrangement of subunits in the terminal complexes. Comparison of *Valonia* and *Boergesenia* suggests a correlation between the TC length and microfibril width, if the nature of the subunit in the TCs is similar in these algae [18., 19]. Similar correlations seem to hold for bacterial cellulose and *Micrasterias*, where greater longitudinal dimensions of the terminal complex are related to wider microfibrils [20].

The distinctive features in microfibril structure and TC shape also seem to be related to the crystal structure. The solid-state ^{13}C-NMR spectra have revealed the existence of two crystalline forms in cellulose I, denoted as cellulose $I\alpha$ and $I\beta$. The algal/bacterial cellulose is $I\alpha$ and higher plant cellulose is a mixture of $I\alpha$ and $I\beta$ [21]. Apparently, cellulose $I\alpha$ is related to highly crystalline cellulose produced by linear TCs, and cellulose $I\beta$ to small and paracrystalline microfibrils produced by solitary rosettes. The situation is summarized in Table I.

"UP" OR "DOWN" IN CELLULOSE I

There are two possible structure for a parallel packing model for cellulose I called parallel-up and parallel-down [1]. They arise from the

way glucan chains are placed in the monoclinic unit cell (which is fixed in space), i.e., with their reducing ends pointing up or down. An alternative way to visualize this is to fix the chain polarity, as if we were looking at the cross section of the microfibril being produced from the synthesizing apparatus, and invert the polarity of the unit cell (Fig. 6)

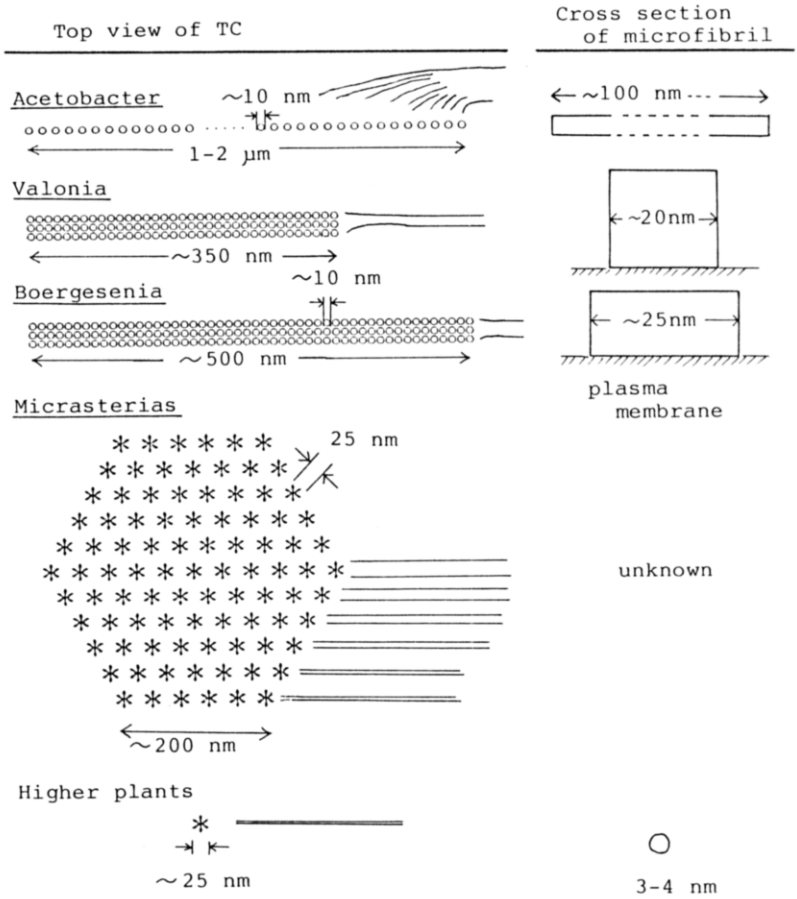

Fig. 5. Schematic drawing of terminal complexes (TCs) and microfibrils. All TCs are arranged to proceed to the left, depositing the microfibrils. TCs and microfibrils are drawn at the same scale in each part except for *Acetobacter*, which has a very long linear TC and a very wide microfibril (ribbon). The hexagonal array of rosettes in *Micrasterias* produces a bundle of microfibrils, each of which corresponds to a row of rosettes [20].

Table I
Correlation between Properties of the Microfibril and TC Morphology

	bacteria	algae		vascular plants
		Siphono-cladales	Zygnema-tales	
Crystallite size	medium	high	?	small
Cross section	flat ribbon	square or rectangular	?	?
Crystallographic orientation	twisted	uniplanar	?	none
Resistance to chemicals	medium	high	?	low
^{13}C-NMR	α	α	?	α + β
TC Shape	linear	linear	hexagonal array of rosettes	isolated rosette

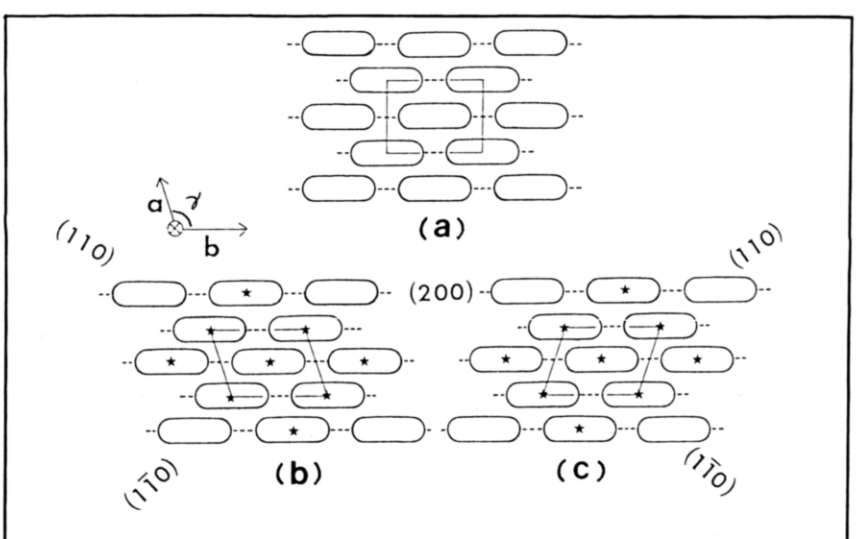

Fig. 6. Schematic diagram of the parallel-up and parallel-down structure of cellulose I. All chains are placed with reducing ends pointing toward the viewer. The hypothetical orthorhombic unit cell (a) is skewed to the left (b), or right (c), resulting in an "up" or "down" structure respectively. Because each chain lacks mirror symmetry, (b) and (c) are not equivalent. Asterices indicate the shape of the crystalline microfibril. The differences in lengths of *a*- and *b*-axes and monoclinic angle are exaggerated. Indexing is based on Ref. 2.

We can start from the imaginary orthorhombic unit cell which has the angle $\gamma = 90°$, as shown in Figure 6a. Because this imaginary structure is unstable (otherwise, this would be the real native cellulose), it has to be skewed to make the actual monoclinic angle, $\gamma = 97°$. Obviously there are two ways of skewing (Figs. 6b and 6c). The "left skew" would make the parallel-up and "right skew" the parallel-down. If the real cellulose I is either one of these two, there has to be some mechanism for selecting it over the other. There seem to be two possible cases as extremes.

i) The nature of the interaction between cellulose molecules in the lattice automatically determines which way to go. This means that there is a significant difference in the energy of stabilization between the "up" and "down" structures. This can be viewed as a "thermodynamic" control mechanism.

ii) Some external physical constraint, such as enzyme configuration, determines the sense of skew. In this case, even an energetically unfavorable form may result. Such a process would be a "kinetic" mechanism.

Though real cellulose molecules are probably crystallized without forming the metastable orthorhombic structure, the basic nature of the selection of "up" or "down" structure could be either "thermodynamic" or "kinetic", or an intermediate of the two.

There is another possibility that the two structures are equally possible and actually occur in nature. That the two structures are hardly distinguishable makes this situation somewhat likely. The two structures may be occurring as alternatives for each organism, or conceivably, some native cellulose might be a mixture of the two.

Here we notice an intriguing possibility that the "up" and "down" structures might be somehow related to the structures $I\alpha$ and $I\beta$, described above. Elucidating the molecular nature and origins of these sets of crystal forms, and also having detailed knowledge about the enzymatic mechanisms of biosynthesis, we may be able to have a unified picture of biocrystallization of cellulose in the near future. Together with progress in the *in vitro* synthesis study, such a knowledge may some day provide us a technology to crease "man-made" cellulose as macroscopic single crystals.

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